Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2014.1003610

57 (2016) 5358–5364
March

Taylor & Francis
Taylor & Francis Group

RP-HPLC and spectroscopic characterization of Suwannee River water NOM after concentrated urea treatment and dialysis

Olga E. Trubetskaya[a], Claire Richard[b,c], Guillaume Voyard[b,c], Victor V. Marchenkov[d], Oleg A. Trubetskoj[e,*]

[a]*Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia, Tel. +7 4967 730859; email: trub@bibch.ru*
[b]*Clermont Université, Institut de Chimie de Clermont-Ferrand, BP 10448, 63000 Clermont-Ferrand, France, Tel. +33 473407142; email: Claire.Richard@univ-bpclermont.fr (C. Richard), Tel. +33 473407815; email: Guillaume.VOYARD@univ-bpclermont.fr (G. Voyard)*
[c]*Equipe Photochimie CNRS, UMR 6296, ICCF, F-63171 Aubière, France*
[d]*Institute of Protein Research, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia, Tel. +7 4967 318272; email: vmarch@rambler.ru*
[e]*Institute of Basic Biological Problems, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia, Tel. +7 4967 733136; email: olegi03@yahoo.com*

Received 5 June 2014; Accepted 26 December 2014

ABSTRACT

Suwannee River natural organic matter (SRNOM) was treated by 7 M urea and then purified by dialysis on 10 kDa membrane. The untreated SRNOM and treated (USRNOM) samples were examined using UV–visible and fluorescence spectroscopies and reversed-phase high-performance liquid chromatography (RP-HPLC) with online absorbance and fluorescence detection. USRNOM was 1.5-fold more absorbing at 280 nm than SRNOM and four fold less fluorescent than SRNOM upon excitation at 270 nm. RP-HPLC analyses of the two samples revealed that USRNOM was somewhat more hydrophobic than SRNOM and both samples contained at least two groups of HS-like fluorophores with different hydrophobicity and protein-like fluorophore(s). Data indicate that protein-like fluorophores were not lost during dialysis. They showed hydrophobic properties and seemed highly fluorescent. HS-like and protein-like fluorophores from water NOM could be successfully separated by RP-HPLC. This raises the prospect of their further research and identification and could be significant for future NOM chemical structure characterization.

Keywords: Purification by dialysis; Urea; Water NOM; RP-HPLC; UV–visible and fluorescence spectroscopies

1. Introduction

Natural organic matter (NOM) is one of the main environmental protective components of our planet, which provide many ecosystem functions by binding and inactivating pesticides, herbicides, heavy metals, and other pollutants. NOM is mandatory and refractory organic component of natural water sources. The light absorption by these substances increases exponentially with decreasing wavelength across the

*Corresponding author.

visible and ultraviolet spectrum, providing aquatic organisms protection from damaging ultraviolet radiation and altering the quantity and quality of light available for photosynthesis. A major fraction of NOM in most waters is humic substances (HS), which is composed of a variety of chemical functional groups and ensure their hydrophobic/hydrophilic and optical properties.

It is well-known that high concentrations of urea are commonly used in biochemistry to disrupt noncovalent bonds in proteins [1]. Meanwhile, concentrated urea has been used, when modern analytical techniques such as polyacrylamide gel electrophoresis, ultrafiltration, and low- and high-pressure size exclusion chromatography were applied for NOM fractionation [2–7]. Since urea is known to form complex with nonionic detergents [8], it may form complexes between urea and nonionic NOM compounds and we can assume that it could lead to some changing of physical–chemical properties of NOM. On this reason NOM entities, obtained during fractionation in the presence of concentrated urea, should be cleared from urea before their further investigation. One method that is widely used to purify NOM samples from urea is a dialysis. Recently, some preliminary results have been obtained with using ^{1}H NMR technique, which showed some structural differences between the initial soil HS and the urea-treated HS samples [9].

Fractionation of NOM according to their hydrophobicity is a common procedure in the study of this polydispersed complex natural mixture, and in the last 25 years, reversed-phase high-performance liquid chromatography (RP-HPLC) was used for the comparison of the hydrophobic/hydrophilic properties of water NOM from different sources [10–15]. Changing hydrophobic/hydrophilic properties of NOM during the dialysis process may vary NOM impact assessment on the transport of organic pollutants and other processes. However, we are not aware of any literature data about the characterization of hydrophobic/hydrophilic properties of NOM, which have been dialyzed after concentrated urea treatment.

Water NOM is intensively fluorescent, and fluorescence detection at a specific excitation/emission (Ex/Em) wavelength has been employed in HPLC separation and characterization of NOM [14,16]. However, the literature data about fluorescent properties of urea-treated NOM after dialysis are very scarce. We could mention the work of Trubetskoj et al. [17], who found that after dialysis from 7 M urea on 5 kDa cellulose membrane, the fluorescence intensity of Suwannee River NOM (SRNOM) was considerably less, than that for non-dialyzed sample. Further determination of distribution of fluorescent constituents between NOM fractions of different polarity and urea-treated NOM samples could be promising for understanding of nature of water NOM fluorescence.

The objectives of the current work are as follows: (i) to use RP-HPLC with online absorbance and fluorescence detection for analysis of SRNOM and dialyzed urea-treated Suwannee River NOM (USRNOM); (ii) to look at the relationship between fluorescence and hydrophobicity of SRNOM and USRNOM. Suwannee River was selected as one of the standards of the International Humic Substances Society (IHSS).

2. Materials and methods

2.1. Materials

The NOM sample, isolated by reverse osmosis from Suwannee River, Georgia, USA (SRNOM), was purchased as a standard material from the IHSS (ref. number 1R101 N). Major elemental composition of SRNOM was as follows: C 52.5%, H 4.2%, and N 1.1%. Water was purified using a Millipore Milli-Q system (Millipore αQ, resistance 18 MΩ cm^{-1}, DOC < 0.1 mg L^{-1}). Phosphate buffer (10 mM or 30 mM Na_2HPO_4/ NaH_2PO_4, pH 6.5) was prepared on reversed osmoses water, obtained with RIOS 5 and Synergy, Millipore. The choice of buffer was due to its large buffer capacity and wide application in studies of HS by RP-HPLC. Urea was purchased from Sigma-Aldrich (Moscow, Russia).

2.2. SRNOM urea treatment and dialysis

To investigate the effect of urea treatment and dialysis on SRNOM physical–chemical and structural properties, 100 mg of the sample was dissolved in 7 M urea solution for 2 d and dialyzed for 7 d against distilled water using cellulose dialysis tubing with nominal cut-off 10 kDa (Sigma-Aldrich, Moscow, Russia), then lyophilized and used for further physical–chemical analyses.

2.3. High-performance size exclusion chromatography

High-performance size exclusion chromatography (HPSEC) of SRNOM and USRNOM samples were performed on a Waters high-performance liquid chromatographic system with Separation Module 2695 (Waters Corp., Milford, MA, USA). The absorbance detection at 270 nm was carried out using a Photodiode array detector 2998 (PDA) working in the range 210–400 nm (Waters Corp., Milford, MA, USA). An analytical column TSKgel $G2000SW_{XL}$, 7.8 mm ID × 300 mm L, particle size 5.0 μm, equipped a guard

column SW_{XL}, 6.0 mm ID × 40 mm L, (TOSOH Bioscience, Japan) was used as stationary phase. The 30 mM phosphate buffer, pH 6.5, was used as a mobile phase and for sample dissolution. SRNOM and USRNOM samples were prepared exactly in the same manner: the absorbance of each sample was adjusted to 6.5 a.u at 270 nm in a 1 cm quartz cuvette on the basis of diluted solutions. The volume of injection onto the TSKgel column was 0.02 mL for both samples. The column was maintained at 30˚C. Flow rate was 1.0 mL min^{-1}. The void column volume (V_o = 5.4 mL) was determined with blue dextran 2000. The total column volume (V_t = 15.43 mL) was calculated on the basis of ID and L of the column and guard column. Analysis of 30 mM phosphate buffer, pH 6.5, was performed before the analyses of the SRNOM and USRNOM to check the absence of any absorbance peaks in the mobile phase. The entire cycle of HPSEC procedure was repeated three times. Deviations between three chromatogram's profiles of each sample did not exceed 3.0%.

2.4. RP-HPLC with online absorbance and fluorescence detection

SRNOM and USRNOM analyses were performed on a Waters ACQUITY™ ultra-performance liquid chromatographic system with cooling auto sampler (Acquity Sample Manager), binary pumping module (Acquity Solvent Manager) and column oven enabling temperature control of analytical column (Waters Corp., Milford, MA, USA). UV–visible detection was carried out using an acquity PDA detector working in the range 210–400 nm. The fluorescence of samples was monitored using an acquity fluorescence detector (FLR), the excitation wavelength was set at 270 nm, and the emission range was 300–600 nm. The fluorescence FLR detector was connected directly to the waste line of the absorbance PDA detector. Both detectors were from Waters Corp. (Milford, MA, USA). The delay between PDA and FLR detection was 0.12 min. Data were collected and processed by chromatographic software Empower2™ (Waters Corp., Milford, MA, USA). An analytical column (UPLC© BEH C18, 2.1 mm ID × 100 mm L, particle size 1.7 μm—Waters Corp., USA) was used as stationary phase. The column was maintained at 30˚C. The solvent A (methanol, HPLC grade Chromasolv®, Sigma-Aldrich) and solvent B (10 mM phosphate buffer, pH 6.5) were used for gradient formation. A stepwise gradient was applied starting at 0.00 min with 0% of solvent A in solvent B, at 2.22 min—10% A in B, at 3.33 min—20% A in B, at 4.45 min—30% A in B, at 5.56 min—40% A in B, at 6.67 min—50% A in B, at 7.7 min—60% A in B, at 11.12 min—70% A in B, at 13.89 min—100% A, at 20 min—0% A in B and continuing for another 10 min at flow rate of 0.3 mL min^{-1}. Solutions of NOM were prepared by dissolving weighed amounts of dry material in 10 mM phosphate buffer, pH 6.5. To estimate the relative amounts of NOM eluted during the RP-HPLC analyses (or conversely, the relative amount being adsorbed on the column), all samples were prepared exactly in the same manner: the absorbance of each sample was adjusted to 6.5 a.u at 270 nm in a 1 cm quartz cuvette on the basis of diluted solutions. The volume of injection onto the reverse-phase column was 0.005 mL for each sample. All NOM matter, which did not adsorb on the column, was eluted from the column during first 10 min. Flow rate was 0.3 mL min^{-1}. Total peaks area was determined using software Empower2™ (Waters Corp., Milford, MA, USA) and compared. Analyses of 10 mM phosphate buffer were performed before the analyses of the NOM samples to check the absence of any fluorescent and absorbance peaks in the solvent. The entire cycle of RP-HPLC procedures was repeated three times. Deviations between three chromatogram's profiles of each sample did not exceed 3.0%.

2.5. UV–visible absorption and fluorescence emission spectra

UV–visible absorption spectra of SRNOM and USRNOM were recorded using a Cary 3 spectrophotometer (Varian, Cary, USA) in a 1 cm quartz cuvette at a concentration 50 mg L^{-1}, from 200 to 700 nm in 10 mM phosphate buffer, pH 6.5. Fluorescence emission spectra of SRNOM and USRNOM were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian, Cary, NC, USA) in a 1 cm quartz cuvette. Excitation wavelength was 270 nm, and emission spectra were recorded from 310 to 700 nm. In order to minimize the inner filter effects, the solutions were diluted with distilled water to absorbance of 0.05 ± 0.01 at 270 nm. UV–visible absorption spectra and fluorescence emission spectra of the RP-HPLC peaks were extracted from the data of PDA- and FLR-detectors. The ratio of the absorbances at 270 nm and 366 nm (A270/A366) was calculated and showing the different slope of absorption spectra of SRNOM, USRNOM and RP-HPLC peaks. The selection of this ratio is due to the PDA detector working parameters (210–400 nm) and the limitations the calculation program of the equipment used. The measurement errors were 1% or less.

3. Results and discussion

3.1. UV–visible absorption and fluorescent emission spectra of SRNOM and USRNOM

The standard SRNOM sample was completely dissolved in 7 M urea solution and dialyzed against distilled water using dialysis tubing with nominal cut-off 10 kDa. Upon urea treatment and dialysis, the SRNOM lost about 60% of the original weight. Fig. 1 shows UV–visible absorption spectra of SRNOM and USRNOM. Both spectra were featureless. The absorption decreased gradually with increasing wavelength, but after dialysis the specific absorption coefficient at 280 nm (A280) increased by a factor of 1.5 (from 0.62 to 0.92). Increase at all wavelengths absorption after urea treatment and dialysis can be attributed to the loss of low molecular size (MS) and weakly absorbed SRNOM matter.

Fluorescence emission spectra of SRNOM and USRNOM recorded upon excitation at 270 nm are given in Fig. 2. They resulted in broad emission bands with maxima at about 455 and 478 nm, respectively. The maximum of emission for USRNOM was red-shifted by 23 nm compared to that of SRNOM and the fluorescence intensity at the maximum was fourfold lower than for SRNOM. The 7 M urea seems to break H-bonds between constituents of NOM components and thus changed the MS distribution. For the validation of this suggestion, SRNOM and USRNOM were characterized by HPSEC in 30 mM phosphate buffer, pH 6.5 (Fig. 3). Both chromatograms show one main broad peak, but the maximum of the elution curve of USRNOM is shifted to shorter retention time vs. SRNOM. This data clearly shown redistribution in toward of increasing the content of high MS components in USRNOM vs. SRNOM. Thus we can

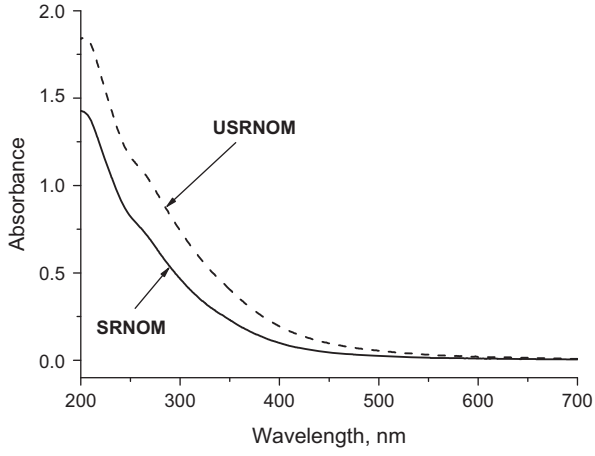

Fig. 1. UV–visible absorption spectra of SRNOM and SRUNOM samples at $C = 50$ mg L^{-1}.

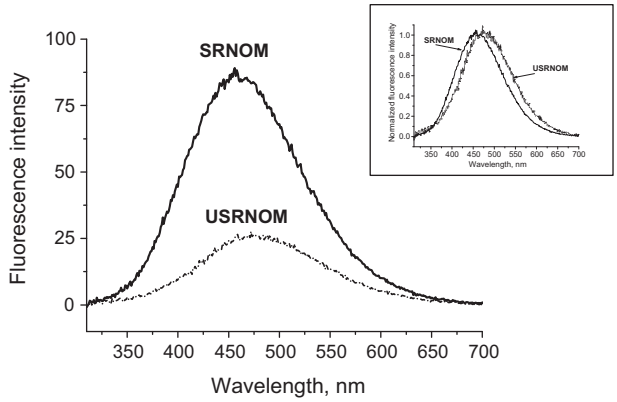

Fig. 2. Emission spectra of SRNOM and USRNOM samples, $\lambda_{ex} = 270$ nm and A270 = 0.05. Inset: spectra normalized at the emission maxima.

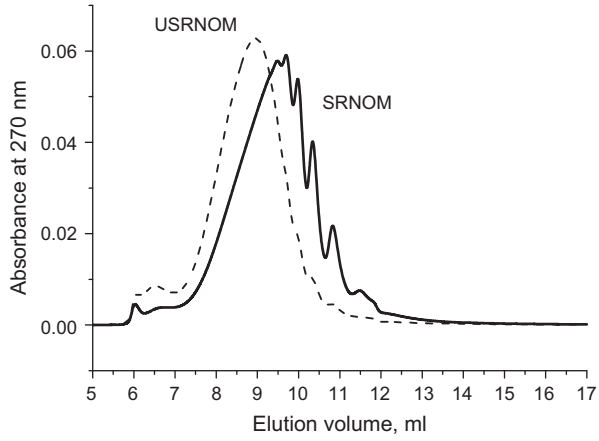

Fig. 3. HPSEC chromatograms of SRNOM (——) and USRNOM (- - - -) with online absorbance at 270 nm. Samples (0.02 mL) with optical density 6.5 a.u were injected onto the column.

conclude that during urea treatment and dialysis SRNOM lost the material with nominal MS less than 10 kDa which weakly absorbed UV-light and enriched in short-wavelength fluorophores.

3.2. RP-HPLC of SRNOM and USRNOM with online absorbance and fluorescence detection

Fig. 4(a) and (c) presents chromatograms of SRNOM and USRNOM obtained by RP-HPLC with online absorbance detection at 270 nm. Both chromatograms exhibited the resolution of six peaks. The hydrophobicity of peaks increased from the first eluted peak to the sixth due to the increasing of methanol concentration, used in the stepwise separation procedure. For simplification, we refer to the first peak

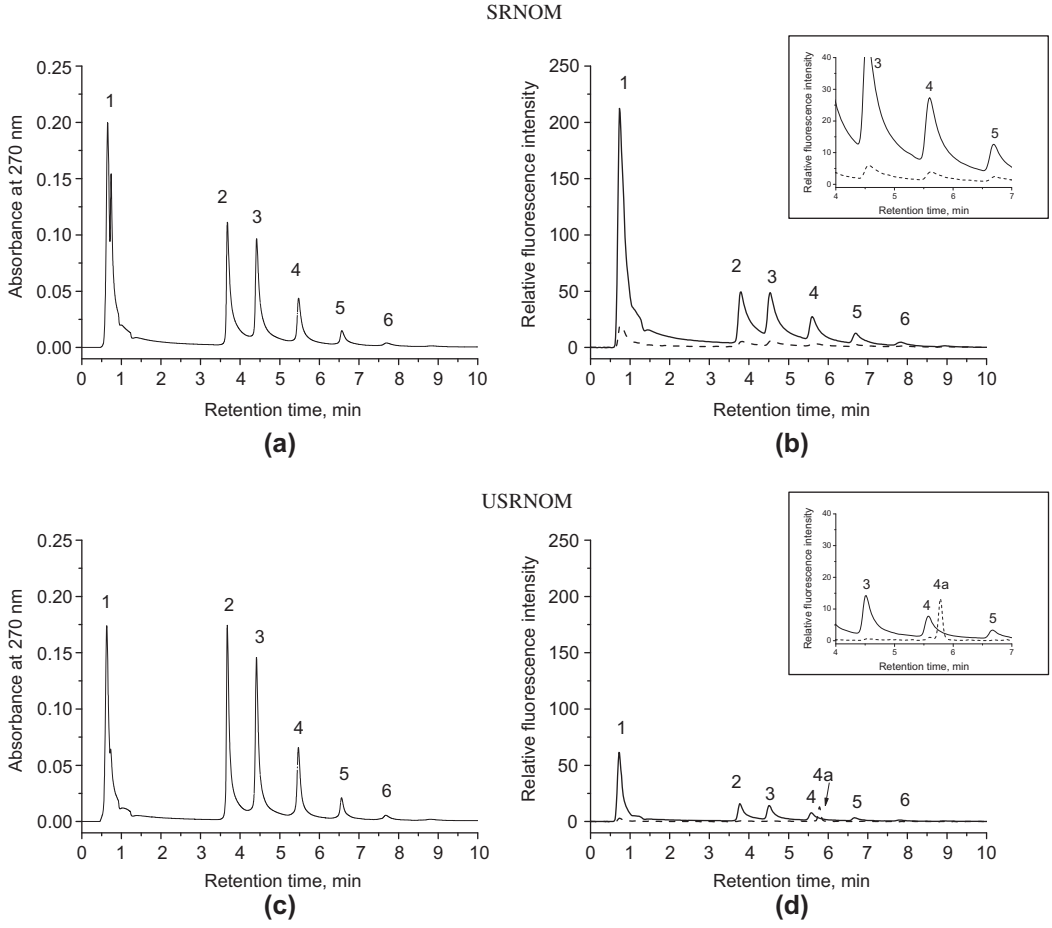

Fig. 4. RP-HPLC chromatograms with absorbance detection at 270 nm for SRNOM (a) and USRNOM (c) and fluorescence detection for SRNOM (b) and USRNOM (d), $\lambda_{ex} = 270$ nm, $\lambda_{em} = 450$ nm (——) and $\lambda_{em} = 330$ nm (- - - -). Samples (0.005 mL) with optical density 6.5 a.u were injected onto the column.

as hydrophilic, because it was eluted in an aqueous phosphate buffer only, whereas the other peaks (2–6) are assumed as hydrophobic, because they were eluted by methanol at different concentrations (from 10 to 50%). Peak 6, eluted by 50% methanol, had the highest hydrophobicity. No material was eluted by methanol at a concentration more than 50% in both samples investigated. Total peaks area was similar between SRNOM and USRNOM, thus the relative recovery of both samples was identical. However, we cannot evaluate the exact amount of NOM material remained adsorbed on the column. Table 1 shows the relative contribution (%) of hydrophilic and hydrophobic peaks, calculated on the basis of area of peaks 1–6. SRNOM had a relatively higher abundance of hydrophilic constituents: 53% of the total peaks area corresponding to peak 1 and areas under hydrophobic peaks 2–6 essentially decreased with the increasing of methanol concentration. Meanwhile, USRNOM had a lower content of hydrophilic components, peak 1 covers only 38% of total peaks area (Fig. 4(a), (c) and Table 1). These results demonstrate that USRNOM is somewhat more hydrophobic than SRNOM.

The absorption spectra of RP-HPLC peaks 1–5 of SRNOM and USRNOM in the region 210–366 nm were extracted from the data of PDA detector. They showed a gradual decrease in absorbance with increasing wavelength without any distinct maximum, as generally observed for NOM. However, differences appeared in the values of absorbance ratio A270/A366 (Table 1). For peak 6, this ratio could not be calculated due to the very low concentration of NOM at this retention time. The A270/A366 values for bulk SRNOM and USRNOM were between those for hydrophilic peak 1 and hydrophobic peak 2. The differences between A270/A366 values of hydrophobic peaks 2–5 were not statistically significant and differ greatly for those values of hydrophilic peaks in both samples.

Table 1
Relative contribution of resolved peaks on RP-HPLC total chromatograms, absorbance ratio A270/A366 of SRNOM and USRNOM and corresponded peaks 1–6, 4a and fluorophoric emission maxima at $\lambda_{ex} = 270$ nm

Sample	Relative contribution (%) of RP-HPLC resolved peaks 1–6	A270/A366	Fluorophoric emission maximum (nm), $\lambda_{ex} = 270$ nm		
			Protein-like	Hydrophilic HS-like	Hydrophobic HS-like
SRNOM	–	3.89			
Peak 1	53	5.26		435	
Peak 2	19	3.25			450
Peak 3	15	3.10			450
Peak 4	8	3.37			450
Peak 5	4	3.07			450
Peak 6	1	n.d.[a]		n.d.[a]	
Σ Peaks 2–6	47				
USRNOM		3.17			
Peak 1	38	3.71		445	
Peak 2	27	3.05			460
Peak 3	21	2.93			460
Peak 4	9	2.87			460
Peak 4a	n.d.[b]	n.d.[b]	330		
Peak 5	4	2.85			460
Peak 6	1	n.d.[a]		n.d.[a]	
Σ Peaks 2–6	62	–			

[a]n.d.—The ratio A270/A366 and fluorescence emission maxima were not determined due to the very low concentration of NOM at the retention time, corresponding for the peak 6 of SRNOM and USRNOM.
[b]n.d.—Relative contribution (%) and the ratio A270/A366 were not determined due to the very low concentration of NOM at the retention time, corresponding to peak 4a of USRNOM.

The fluorescence signals in water NOM can be generally classified into two types of fluorophoric groups. One group usually has excitation (Ex) maximum less than 305 nm and emission (Em) maximum less than 380 nm, which is related to aromatic amino acids, and is often referred to as protein-like fluorescence [16]. The other group with Ex maximum ranging from 220 to 360 nm and Em maximum wavelengths ranging from 380 to 470 nm are attributed to HS of water NOM [16,18]. On this reason for detection of both groups of fluorophores in one chromatographic experiment, we used excitation wavelength at 270 nm.

Fig. 4(b) and (d) presents RP-HPLC chromatograms of SRNOM and USRNOM with online fluorescence detection at excitation–emission wavelength pairs (Ex/Em) 270/450 nm and 270/330 nm for detection of HS- and protein-like fluorophoric groups, respectively. The SRNOM and USRNOM chromatograms at 270/450 nm revealed fluorescence in peaks 1–5 with highest intensity in hydrophilic peak 1 and a general decrease from peak 2 to peak 5. In peak 6 of SRNOM and USRNOM, a very weak fluorescence was measured and it was not taken into consideration. The maximum of emission in SRNOM was 435 nm for hydrophilic peak 1 and 450 nm for hydrophobic peaks 2–5 (Table 1). In USRNOM, peak 1 had fluorescence emission maximum at 445 nm, while hydrophobic peaks 2–5 revealed emission maximum at 460 nm (Table 1). However, it should be noted that bulk SRNOM and USRNOM had maximum of fluorescence emission at 455 nm and 478 nm, respectively (Fig. 2). Perhaps the part of long-wavelength fluorophores located in highly hydrophobic part of SRNOM irreversibly adsorbed on the C18-column and were not subjected to RP-HPLC characterization.

The fluorescence emission with maximum at 330 nm, which is related to aromatic amino acids and is often referred to as protein-like fluorescence, was not revealed in a bulk SRNOM extracted and purified by IHSS method, as reported by other researchers [14,18] and very poorly expressed in USRNOM (Fig. 2, inset). However, a peak 4a with a strong protein-like fluorescence emission ($\lambda_{max} = 330$ nm) occurred on RP-HPLC chromatogram of dialyzed USRNOM at retention time 5.78 min (Fig. 4(d), inset, Table 1). Interestingly, this peak was not observed in chromatogram of SRNOM (Fig. 4(b), inset). These results suggest that the protein-like fluorophore(s) was not lost during dialysis and possess hydrophobic properties. The urea treatment seems to release protein-like compounds that were held by H-bonds in NOM structure. This

explains that protein-like peak was not observed in SRNOM. Moreover, it should be noted that at the retention time 5.66 min (the delay between PDA and FLR detection was 0.12 min) no absorbtion at 270 nm was detected (Fig. 4(c)). That means that fluorophore (s) with emission maximum at 330 nm possesses extremely high quantum yield vs. HS-like fluorophore(s), located in peaks 1–6.

4. Conclusions

Our data indicate that after dialysis, SRNOM is somewhat more hydrophobic, which may affect the conclusions about the functions of organic matter in the natural waters. Successful separation of SRNOM, before and after dialysis, using RP-HPLC with online absorbance and fluorescence detection showed that: (i) SRNOM contained at least two groups of different HS-like fluorophores with different hydrophobicity and emission maxima (Table 1); (ii) protein-like fluorophore (s) was not lost during dialysis, possesses strongly hydrophobic properties and is highly fluorescent.

The identification of HS-like and protein-like fluorophores in USRNOM by RP-HPLC separation has been realized and raises the prospect of their further research. The data obtained could be significant for future NOM chemical structure characterization.

Acknowledgements

The work has been supported by Russian Foundation for Basic Research (projects no 13-05-00241_a and 15-04-00525_a).

References

[1] M. Oh-Ishi, T. Maeda, Separation techniques for high-molecular mass proteins, J. Chromatogr. B 771 (2002) 49–66.
[2] M. Castagnola, R.G. De Las Heras, G.B. Marini-Bettolo, C. Nigro, Effect of urea on electrophoretic pattern of soil humic acids, J. Chromatogr. A 147 (1978) 438–442.
[3] O.A. Trubetskoj, O.E. Trubetskaya, O.I. Reznikova, G.V. Afanasieva, C. Saiz-Jimenes, Polyacrylamide gel electrophoresis of soil humic acid fractionated by size exclusion chromatography and ultrafiltration, J. Chromatogr. A 767 (1997) 285–292.
[4] A. Piccolo, The supramolecular stucture of humic substances, Soil Sci. 166 (2001) 810–832.
[5] J. Peuravuori, V. Lepane, T. Lehtonen, K. Pihlaja, Comparative study of separation of aquatic humic substances by capillary zone electrophoresis using uncoated, polymer coated and gel filled capillaries, J. Chromatogr. A 2004 (1023) 129–142.
[6] O.E. Trubetskaya, L.A. Shaloiko, D.V. Demin, V.V. Marchenkov, I.I. Proskuryakov, C. Coelho, O.E. Trubetskoj, Combining electrophoresis with detection under light and multiple ultrafiltration for isolation of humic fluorescence fractions, Anal. Chim. Acta 690 (2011) 263–268.
[7] S. Karim, M. Aoyama, Fractionation of soil humic acids by preparative polyacrylamide gel electrophoresis in the presence of concentrated urea, Soil Sci. Plant Nutr. 59 (2013) 827–839.
[8] L.M. Hjelmeland, Solubilization of native membrane proteins, in: M.P. Deutsher (Ed), Guide to Protein Purification, Methods of Enzymology, 1990, San Diego, Calif. Academic Press, pp. 253–264.
[9] O.A. Trubetskoj, P.G. Hatcher, O.E. Trubetskaya, ^{1}H-NMR and ^{13}C-NMR spectroscopy of chernozem soil humic acid fractionated by combined size-exclusion chromatography and electrophoresis, Chem. Ecol. 26 (2010) 315–325.
[10] F.Y. Saleh, W.A. Ong, Structural features of aquatic fulvic acids. Analytical and preparative reversed-phase high-performance liquid chromatography separation with photodiode array detection, Anal. Chem. 61 (1989) 2792–2800.
[11] A.T. Lombardi, W.F. Jardim, Fluorescence spectroscopy of high performance liquid chromatography fractionated marine and terrestrial organic materials, Water Res. 33 (1999) 512–520.
[12] K. Egeberg, J.J. Alberts, Determination of hydrophobicity of NOM by RP-HPLC, and the effect of pH and ionic strength, Water Res. 36 (2002) 4997–5004.
[13] E. Parlanti, B. Morin, L. Vacher, Combined 3D-spectrofluorometry, high performance liquid chromatography and capillary electrophoresis for the characterization of dissolved organic matter in natural waters, Org. Geochem. 33 (2002) 221–236.
[14] F.C. Wu, R.D. Evance, P.J. Dillon, Separation and characterization of NOM by high-performance liquid chromatography and on-line three-dimensional excitation emission matrix fluorescence detection, Env. Sci. Tech. 37 (2003) 3687–3693.
[15] A.C. Stenson, Reversed-phase chromatography fractionation tailored to mass spectral characterization of humic substances, Env. Sci. Tech. 42 (2008) 2060–2065.
[16] P. Coble, Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy, Mar. Chem. 51 (1996) 325–346.
[17] O.A. Trubetskoj, O.E. Trubetskaya, C. Richard, Photochemical activity and fluorescence of electrophoretic fractions of aquatic humic matter, Water Resour. 36 (2009) 543–550.
[18] J.J. Alberts, M. Takacs, Total luminescence spectral characteristics of IHSS standard and reference fulvic acids, humic acids and natural organic matter: Comparison of aquatic and terrestrial source terms, Org. Geochem. 35 (2004) 243–256.